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Optimization of the MHC Class I Peptide Cargo Is Dependent on Tapasin

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Summary

The loading of MHC class I molecules with their peptide cargo is undertaken by a multimolecular peptide loading complex within the endoplasmic reticulum. We show that MHC class I molecules can optimize their peptide repertoire over time and that this process is dependent on tapasin. Optimization of the peptide repertoire is both quantitatively and qualitatively improved by tapasin. The extent of optimization is maximal when MHC class I molecules are allowed to load within the fully assembled peptide loading complex. Finally, we identify a single natural polymorphism (116D>Y) in HLA-B*4402 that permits tapasin-independent loading of HLA-B*4405 (116Y). In the presence of tapasin, the tapasin-independent allele B*4405 (116Y) acquires a repertoire of peptides that is less optimal than the tapasin-dependent allele B*4402 (116D).

Introduction

MHC class I molecules consist of three components: heavy chain (HC), β 2-microglobulin (β_2m), and peptide. The polymorphic heavy chain and nonpolymorphic β_2m initially form a MHC class I heterodimer through noncovalent interactions. The assembly of MHC class I heterodimers with peptides is a prerequisite for both their molecular stability and function in presenting cellular peptides to cytotoxic T cells (Elliott et al., 1991; Townsend et al., 1986). Optimal peptides for individual MHC class I heterodimers are normally 8–10 amino acids in size with specific anchor motifs (Falk et al., 1991). The processes governing the loading of peptide-receptive MHC class I molecules with peptides of appropriate motif and length are orchestrated by a number of ER resident chaperones. These chaperones include TAP (transporter associated with antigen processing), calreticulin, ERp57, calnexin, and tapasin (Cresswell et al., 1999). This multimolecular machinery has been termed the peptide loading complex. While the function of TAP in transporting peptides from the cytosol to the ER has been established, the contribution to peptide loading of the other components is less well understood.

Previous work, undertaken on the tapasin-deficient cell line 721.220 (Grandea et al., 1995; Peh et al., 1998) and more recently on tapasin knockout mice (Garbi et al., 2000; Grandea et al., 2000), has shown tapasin to be integral to efficient MHC class I antigen presentation. Tapasin has been shown to be important for increasing TAP levels (Lehner et al., 1998) and peptide binding to TAP (Li et al., 2000), consequently increasing peptide supply to the ER. Second, it has been identified as a cornerstone of the peptide-loading complex, allowing MHC class I-β_2m heterodimers, calreticulin, and ERp57 to congregate at the TAP interface (Ortmann et al., 1997). Third, a considerable amount of evidence has accumulated over the past few years to suggest that tapasin promotes the optimal peptide loading of MHC class I molecules (Grandea and Van Kaer, 2001).

In this study, we have addressed the concept of progressive peptide optimization for MHC class I antigen presentation. By utilizing the known correlation between the thermostability of MHC class I peptide complexes and the affinity of their peptide cargo (Bouvier and Wiley, 1994; Fahnestock et al., 1992; Schumacher et al., 1990), we demonstrate biochemically the changing peptide profile of a cohort of newly synthesized MHC class I molecules over time within cells. This time-dependent optimization can occur for some alleles in the absence of tapasin, albeit inefficiently. In the presence of tapasin there is a kinetic, quantitative, and qualitative improvement in the peptide repertoires of the MHC class I molecules. This optimization of the peptide repertoire is maximal when MHC class I molecules are loaded in the presence of full-length tapasin, which is able to bridge MHC class I molecules to the TAP interface. Finally, we have identified a single amino acid residue at position 116, which distinguishes HLA-B*4402 from HLA-B*4405 and allows B*4405 to load peptides independently of tapasin. However, the consequence of this natural polymorphism and associated tapasin independence is incomplete optimization when tapasin is present.

Results

Tapasin Optimizes the Peptide Selection of HLA-B*4402 and HLA-B*2705

HLA-B*4402 (B4402) is highly dependent upon coexpression of tapasin for peptide loading. In contrast, the peptide loading of HLA-B*2705 (B2705) was reported to be relatively independent of tapasin (Peh et al., 1998). We have made use of the previous experimental obser-

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vation that correlates the thermostability of a MHC class I complex with the affinity of its peptide cargo to investigate the optimization of B2705 and B4402 in tapasin-deficient and tapasin-reconstituted cells. We explored the thermostability of these MHC class I complexes by heating detergent lysates of radiolabeled cells to varying temperatures for a short time, followed by immunoprecipitation of residual MHC class I complexes with the conformation-dependent antibody W6/32 (Neefjes et al., 1993). The amount of conformationally intact MHC class I complexes that survived the heating step was quantified after separation by SDS-PAGE. The thermostability profile of MHC class I complexes was thus taken to reflect the average binding affinity of peptides bound to these complexes.

To validate this idea within our experimental system, we first correlated the thermostability of MHC class I complexes with the binding affinity of various peptides. Metabolically radiolabeled .220.B4402 cells were lysed in the presence or absence of exogenous B4402 binding peptides, prior to heating and immunoprecipitation with W6/32. Figure 1A shows that the index peptide (SEIDTVAKY) improved the thermostability of B4402 complexes such that 63% were stable at 37°C and 55% at 50°C, compared to 10% at 37°C and 0% at 50°C in the absence of peptide. Alanine substitutions at the anchor positions P2 and P9 of the index peptide failed to improve the thermostability of B4402. The N-terminal extended peptide AAASEIDTVAKY gave an intermediate stability profile, consistent with other studies documenting the importance of optimal peptide length. We also used peptides with a range of binding affinities that had been previously determined using an in vitro refolding assay of B4402 and radiolabeled β_2m. The thermostability of B4402 stabilized with these peptides (80% for AEIAAVAKY at 37°C, 55% for ADIAAVAKY, and 10% for AAIAAVAKY) followed the rank order predicted by their binding affinities (DiBrino et al., 1995). For B2705, we used B2705 binding peptides whose binding affinities had been previously characterized by fluorescence spectroscopy (hierarchy: GRAFVTIGK $>$ GRADVTIGK $>$ GRAFVTIGG $>$ GRAFVTIGS) (Dedier et al., 2000). The thermostability profile of B2705 complexes stabilized with these peptides again correlated exactly with the experimentally derived binding affinities (Figure 1B). The variant peptides GRAFVTIGG and GRAFVTIGS with less optimal P9 residues decreased the extent of 50°C heat-resistant complexes considerably from 60% for the index peptide (GRAFVTIGK) to $\sim$ 5%. The N-terminal extended peptide again demonstrated reduced thermostability despite having identical anchor residues with 85% and 15% of complexes returned at 37°C and 50°C, respectively. Therefore, the thermostability profiles of both B4402 and B2705 correlated with the binding affinity of their peptide cargo.

We next determined the thermostability profile of a cohort of newly synthesized B4402 complexes expressed in .220. As expected, the thermostability of these B4402 complexes in the absence of tapasin is poor, with only 50% of complexes surviving at 25°C and 12% surviving at 37°C (Figures 1C and 1D). This is consistent with B4402 molecules having bound a spectrum of suboptimal ligands in the absence of tapasin. In contrast, B4402 molecules loaded in the presence of tapasin are all thermostable at 37°C, with the majority remaining stable at 50°C (Figure 1D). This improvement in thermostability indicates binding of peptides with an affinity at least as high as the index peptide that is a known CTL epitope in a process that is critically dependent upon tapasin. Although the .220 cell line expresses endogenous HLA-C*0102, the mAb W6/32 recognizes this allele weakly (Neisig et al., 1998). Quantitation of the class I alleles immunoprecipitated by W6/32 and separated by IEF showed that 90%–95% of the recovered heavy chain in these experiments was attributable to the transfected B alleles (data not shown).

In the absence of tapasin, B2705 molecules are expressed on the cell surface at levels similar to those expressed in the presence of tapasin. In accordance with the differing steady-state cell surface expression of B2705 and B4402, the thermostability profile of B2705 molecules was greater than that of the B4402 complexes at all temperatures above 4°C in the absence of tapasin (Figure 1E). However, upon the introduction of tapasin, a dramatic improvement in the thermostability profile of these B2705 complexes (Figure 1E) was seen, with 95% of all complexes now stable at 37°C. Evidently, tapasin is required to achieve optimal loading of not only B4402 but also B2705 molecules.

Comparing the Thermostability of Cell Surface B2705 and Intracellular B2705

The finding that the early cohort of B2705 complexes assembled in the absence of tapasin were more thermolabile than those assembled in the presence of tapasin appeared at first to be inconsistent with the observation that the cell surface expression of B2705 was similar in the presence and absence of tapasin. Thus, we compared the thermostability of cell surface B2705 complexes with the thermostability of an early cohort of B2705 complexes in the presence and absence of tapasin (Figure 2). .220.B2705 and .220.B2705.Tapasin transfectants were radiolabeled with either ^{35}S-methionine/cysteine to indicate newly assembled complexes or iodine-125 to indicate cell surface complexes. In the presence of tapasin, the recently formed B2705 complexes were as stable as cell surface B2705 complexes. In the absence of tapasin, however, B2705 complexes that had assembled early in the ER were much less stable than cell surface B2705 complexes. This suggested that optimization of intracellular MHC class I complexes may have occurred over time.

Tapasin Catalyzes the Time-Dependent Optimization of the B4402 Peptide Repertoire

To follow the thermostability profile of MHC class I complexes over time, we pulse labeled .220.B4402 and .220.B4402.Tapasin cells with ^{35}S-methionine/cysteine for 5 min and chased for 2 hr. The number of W6/32 complexes that were stable at 4°C, 37°C, and 50°C was evaluated at each time point in the absence and presence of tapasin respectively (Figures 3A and 3B). Quantification of heavy chain intensities indicated that the majority of B4402 complexes are maintained throughout the chase period in the presence of tapasin (see 4°C in Figure 3D), whereas the complexes are lost in the absence of tapasin (see 4°C in Figure 3C). This loss is

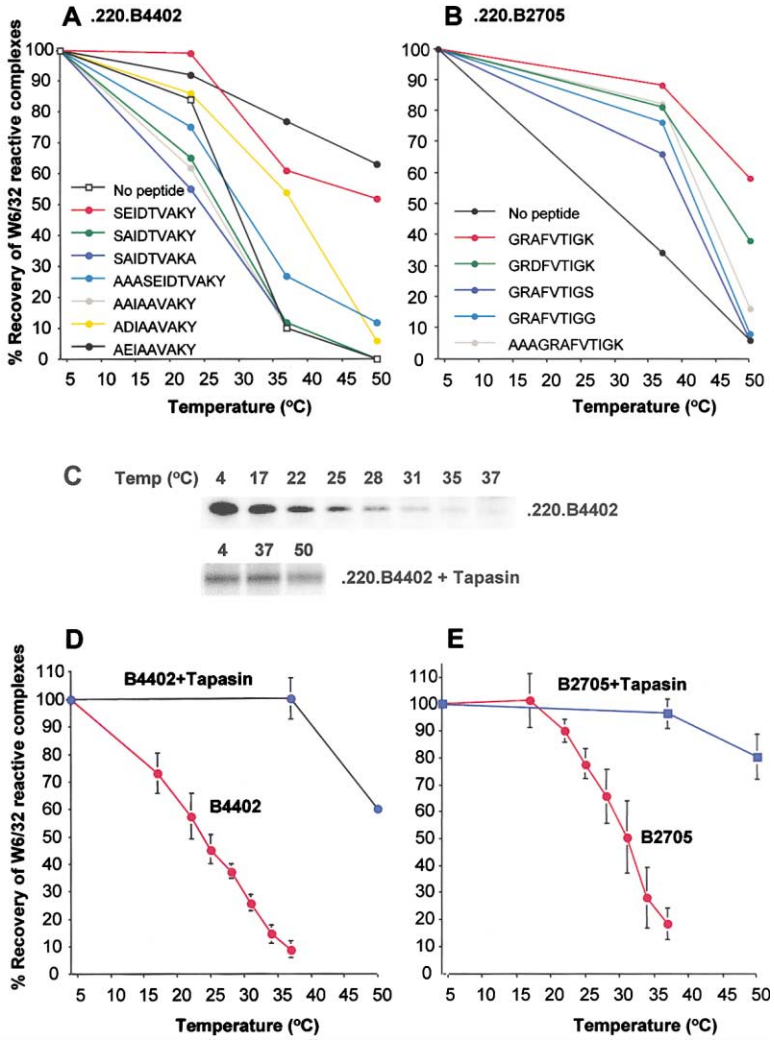

Figure 1. Thermostability of MHC Class I Complexes in the Presence and Absence of Tapasin

(A) .220.B4402 cells were radiolabeled with [^{35}S]-methionine/cysteine for 30 min and lysed in Triton X-100 buffer in the presence or absence of variant synthetic peptides specific for B4402. Equal aliquots were kept at either 4°C or heated at temperatures indicated for 12 min, prior to immunoprecipitation with W6/32. Samples were separated by 10% SDS-PAGE. The intensity of the class I heavy chain radioactive band at each temperature point was quantified by phosphorimaging. The percentage of W6/32 reactive complexes recovered after heating was plotted as the intensity value of class I heavy chain in relation to the intensity value at 4°C. The peptides were SEIDTVAKY (index), SAIDTVAKY (P2 substitution), SAIDTVAKA (P2 and P9 substitution), AAASEIDTVAKY (N-terminal extended), and AAIAAVAKY, ADIAAVAKY, and AEIAAVAKY with half-lives of 18 min, 120 min, and 240 min, respectively, as previously determined by dissociation of incorporated radiolabeled β_2m (DiBrino et al., 1995). Results are representative of at least two experiments.

(B) .220.B2705 cells were radiolabeled for 30 min and lysed in Triton X-100 buffer in the presence or absence of variant synthetic peptides specific for HLA-B2705, prior to heating at temperatures as indicated for 12 min and immunoprecipitation with W6/32. The peptides were GRAFVTIGK (index), GRDFVTIGK, GRAFVTIGS, and GRAFVTIGG, which have Tm (°C) values of 60, 50, 42, and 38, respectively, as determined by circular dichroism spectroscopy (Dedier et al., 2000). AAAGRAFVTIGK represents an N-terminal extended peptide. Results are representative of at least two experiments.

(C) .220.B4402 and .220.B4402.Tapasin cells were radiolabeled with [^{35}S]-methionine/cysteine for 30 min, lysed in Triton X-100, and either kept at 4°C or heated at temperatures indicated for 12 min prior to immunoprecipitation with W6/32. Only class I heavy chains are depicted here.

(D and E) .220.B4402 and .220.B4402.Tapasin cells in (D), and .220.B2705 and .220.B2705.Tapasin cells in (E) were radiolabeled with [^{35}S]-methionine/cysteine for 30 min and then treated as in (C). The intensity of the class I heavy chain radioactive band at each temperature point was quantified by phosphorimaging. The mean intensity value of each band and standard deviations were calculated from triplicate samples. The percentage of W6/32 reactive complexes recovered after heating was plotted as mean intensity value of class I heavy chain in relation to the mean intensity value at 4°C. Results are representative of at least two experiments.

probably due to the dissociation of β_2m following loss of low-affinity peptides, with subsequent loss of the W6/32 epitope. Furthermore, in the absence of tapasin, those B4402 complexes that remained failed to improve their thermostability (Figures 3C and 3E). In contrast, in the presence of tapasin, B4402 complexes became progressively more thermostable over time, having undergone tapasin-dependent and time-dependent peptide optimization (Figures 3D and 3F). Indeed, B4402 complexes had achieved maximal 37°C thermostability by 30 min in the presence of tapasin and continued to optimize their peptide load so that the entire cohort of B4402 complexes reached 50°C thermostability by 120 min. This is consistent with the idea that class I molecules that are unstable at physiological temperature are prone to intracellular degradation and that degradation and optimization are competing processes. Conceivably, optimization may have involved the exchange of suboptimal peptide ligands for ones with higher affinity or trimming of peptide ligands of suboptimal length.

Although we have interpreted the improvement in thermostability to indicate optimization of the B4402 bound peptide cargo, another interpretation might be that previously empty B4402 molecules were being progressively loaded with peptides. We believe this to be unlikely for two reasons. First, B4402 complexes assembled even in the absence of tapasin are unlikely to be empty because coexpression of HSV-1 ICP47 protein in .220.B4402 cells reduced further the thermostability of these B4402 complexes (data not shown and see Figure 5A). ICP47 inhibits TAP-mediated translocation of peptides into the ER (Hill et al., 1995), suggesting that B4402 binds a repertoire of poorly stabilizing peptides in .220 that cannot be optimized in the absence of tapasin.

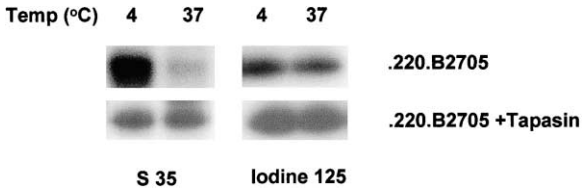

Figure 2. Thermostability of Newly Assembled and Cell Surface HLA-B*2705 Complexes

.220.B2705 and .220.B2705.Tapasin cells were radiolabeled either with [^{35}S]-methionine/cysteine for 30 min to detect newly assembled B2705 complexes or with iodine-125 to probe for cell surface B2705 complexes. After lysis in Triton X-100 buffer, equal aliquots of the lysis supernatant were kept at either 4°C or heated at 37°C for 12 min, followed by immunoprecipitation with W6/32.

Second, we noted that B4402 complexes, which had achieved 37°C thermostability, became progressively resistant to 50°C thermal denaturation over time. Thus at 30 min, 62% of B4402 complexes, which were thermostable at 37°C, were also thermostable at 50°C. This proportion rose to 75% by 60 min and 100% by 120 min. In other words, B4402 complexes that were previously occupied by peptides that conferred 37°C stability continued to optimize their peptide cargo to attain 50°C stability. Additionally, pulse-chase analysis of 220.B4402. Tapasin shows that 44% of the complexes are endoglycosidase H resistant by 30 min (Figure 3G). Therefore, as optimization of B4402 molecules continues between 30 to 120 min, it suggests that post ER mechanisms may exist for the peptide optimization of B4402 complexes.

Tapasin is known to increase the expression of TAP and hence increase the translocation of peptides (Lehner et al., 1998). Therefore, another potential explanation for the superior thermostability of B4402 in the presence of tapasin is that tapasin is required to ensure adequate supply of B4402-specific peptides through TAP. This is unlikely to be the case for several reasons. First, B lymphoblastoid cells are known to constitutively express high levels of TAP (Rowe et al., 1995). Second, interferon-γ treatment of .220.B4402 cells which increased TAP expression and TAP translocation of peptides failed to improve the thermostability of B4402 complexes (data not shown). Third, soluble tapasin which does not change TAP function (Lehner et al., 1998) fully restored cell surface B4402 expression (data not shown and see Figure 6E), and fourth, transfection of an N-terminally truncated tapasin (NΔ44) that stabilized TAP (Bangia et al., 1999) and restored maximal peptide supply was unable to improve the optimization of B4402 (data not shown and F. Momburg, personal communication).

Tapasin-Independent Optimization of the B2705 Peptide Repertoire

The capacity of B2705 complexes to attain 37°C thermostability and traffic to the cell surface in the absence of tapasin despite the fact that newly assembled B2705 complexes are unstable prompted us to investigate whether these complexes may have optimized their peptide cargo over time, independently of tapasin. Thus, .220.B2705 and .220.B2705.Tapasin transfectants were pulse labeled and chased for 2 hr with thermostability evaluated at 30, 60, and 120 min. The thermostability profiles of B2705 complexes from .220.B2705 and .220.B2705.Tapasin transfectants (Figures 4A, 4C, and 4E, and 4B, 4D, and 4F, respectively) show that B2705 complexes could undergo optimization of their peptide load even in the absence of tapasin. This optimization affords an increase of 50°C thermostable material from 10% at 30 min to 50% at 2 hr (Figure 4E). Optimization became maximal at 2 hr, as longer pulse-chase assays showed no further improvement (data not shown). A small proportion of complexes was lost over time in the absence of tapasin, but the magnitude of loss was much smaller compared to 220.B4402. The optimization seen for B2705 in the absence of tapasin (Figures 4A, 4C, and 4E) could represent filling of empty molecules, as not all complexes are 37°C stable at the first time point. However, just as for 220.B4402, the introduction of ICP47 into .220.B2705 cells further destabilized B2705 complexes (Figure 5A). The differences between the thermostability profiles in Figure 5A are evident at all temperatures above 21°C, becoming most marked at 34°C where no complexes are recoverable in the presence of ICP47. Therefore, complexes that are only stable at temperatures below 37°C could be either peptide occupied and undergo optimization or be truly empty and acquire peptides for the first time. Furthermore, B2705 complexes that had attained 37°C thermostability became progressively more resistant to thermal denaturation at 50°C over time. Thus at 30 min, 24% of B2705 complexes which were thermostable at 37°C were also thermostable at 50°C. This proportion rose to 41% by 60 min and 61% by 120 min. The pattern of time-dependent improvement of thermostability is more consistent with peptide-loaded B2705 complexes undergoing optimization of their existing peptide cargo. Indeed, the peptide dependency for the stabilization of B2705 was confirmed by the failure of B2705 complexes to optimize over time in the cell line 220.B2705.ICP47 (Figure 5B). Here the thermostability profile of B2705 compared directly to that of .220.B4402, and the cell surface expression was correspondingly reduced (data not shown). Therefore, B2705 has the ability to load and optimize its peptide repertoire in the absence of tapasin, but this process is reliant upon a supply of TAP-translocated peptides.

The catalytic activity of tapasin was evident even for B2705 (Figures 4D and 4F). By 30 min, the entire cohort of B2705 complexes had achieved 37°C thermostability, greater than that achieved at any time point in the absence of tapasin. At this time point, 75% of the complexes had attained endoglycosidase H resistance and exited the ER (Figure 4G). Furthermore, when brief pulse-chase experiments were performed with a 2 min pulse and 5 min chase periods, approximately 50% of B2705 molecules achieved 50°C stability within 5 min (data not shown). The maximal fraction of B2705 complexes achieving 37°C and 50°C stability in .220.B2705.Tapasin was also far superior to .220.B2705. Thus, tapasin not only provided a kinetic advantage, speeding up peptide loading, but also enhanced the extent of peptide optimization. Significantly, when the optimization profile of

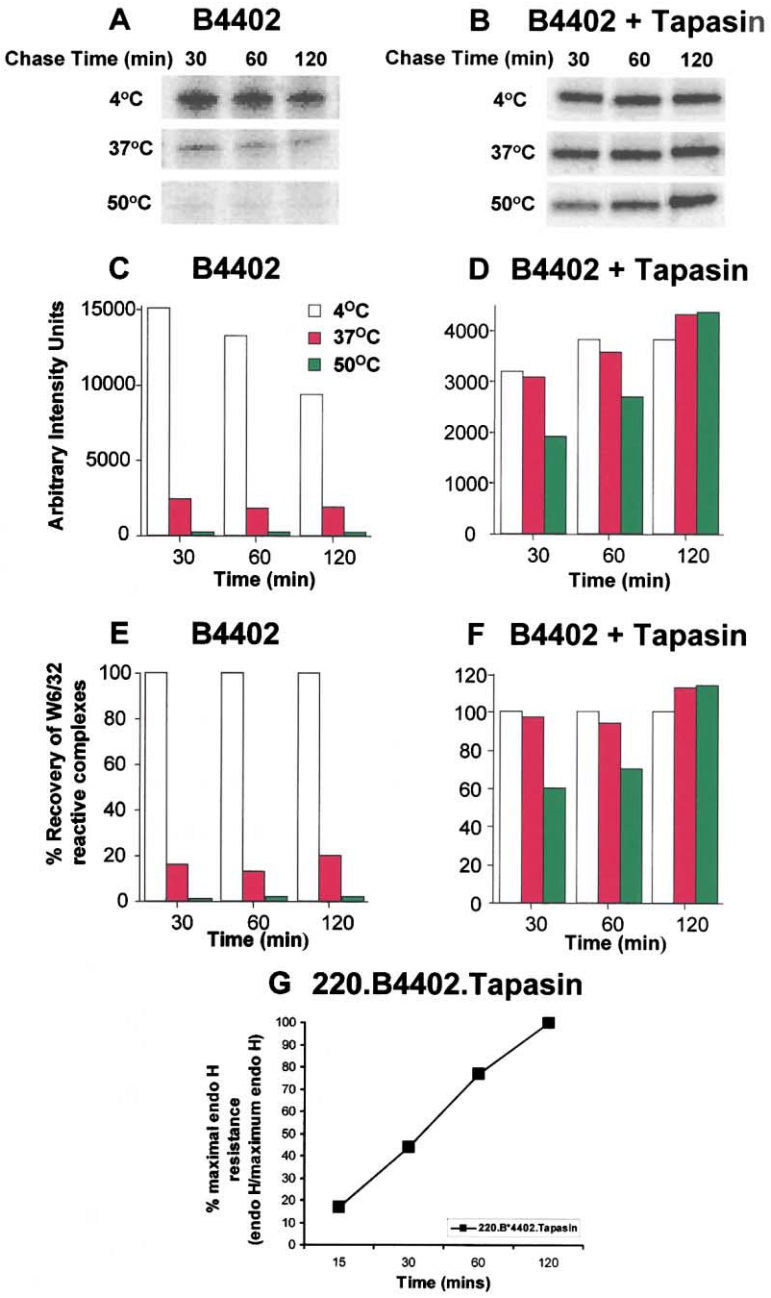

Figure 3. Time-Dependent and Tapasin-Dependent Optimization of HLA-B*4402 Complexes

(A–F) .220.B4402 cells in (A), (C), and (E) and .220.B4402.Tapasin cells in (B), (D), and (F) were pulse labeled with [^{35}S]-methionine/cysteine for 5 min and chased in excess cold methionine/cysteine. Equal numbers of cells were harvested at time points indicated. The samples were then processed and analyzed as in Figures 1C–1E. (A)–(F) are representative of three separate experiments.

(G) 220.B4402.Tapasin cells were labeled for 5 min and chased for 15, 30, 60, and 120 min. At each time point the percentage of endoglycosidase H-resistant material/maximum endoglycosidase H-resistant material is plotted. The results are representative of at least two experiments.

220.B2705.Tapasin (Figure 4F) is compared with .220.B4402.Tapasin (Figure 3F), it is evident that not only does tapasin have a greater impact upon the loading of B4402 with peptides compared to B2705 but the resultant MHC class I peptide complexes in .220.B4402.Tapasin are more thermostable than those in .220.B2705.Tapasin. This was not due to inherent thermostability differences between the two alleles, as both achieved equal thermostability when index peptides were added exogenously (Figures 1A and 1B). It has not been possible to compare alleles in this manner previously, and this finding suggests that there are allelic differences in the extent of optimization that MHC class I molecules may achieve.

Class I-TAP Interaction Is Required for Maximal Peptide Optimization

We wanted to investigate whether the bridging function of tapasin for class I to TAP was relevant for peptide optimization. It has been previously shown that soluble tapasin, lacking its transmembrane and cytoplasmic domains, restored cell surface expression and antigen presentation of HLA-B8, despite the failure of soluble tapasin to restore the interaction between HLA-B8 and TAP (Lehner et al., 1998). We explored this question using .220.B4402.solTapasin and .220.B2705.solTapasin cell transfectants. Indeed, soluble tapasin fully restored the cell surface expression of B4402 (data not shown). We

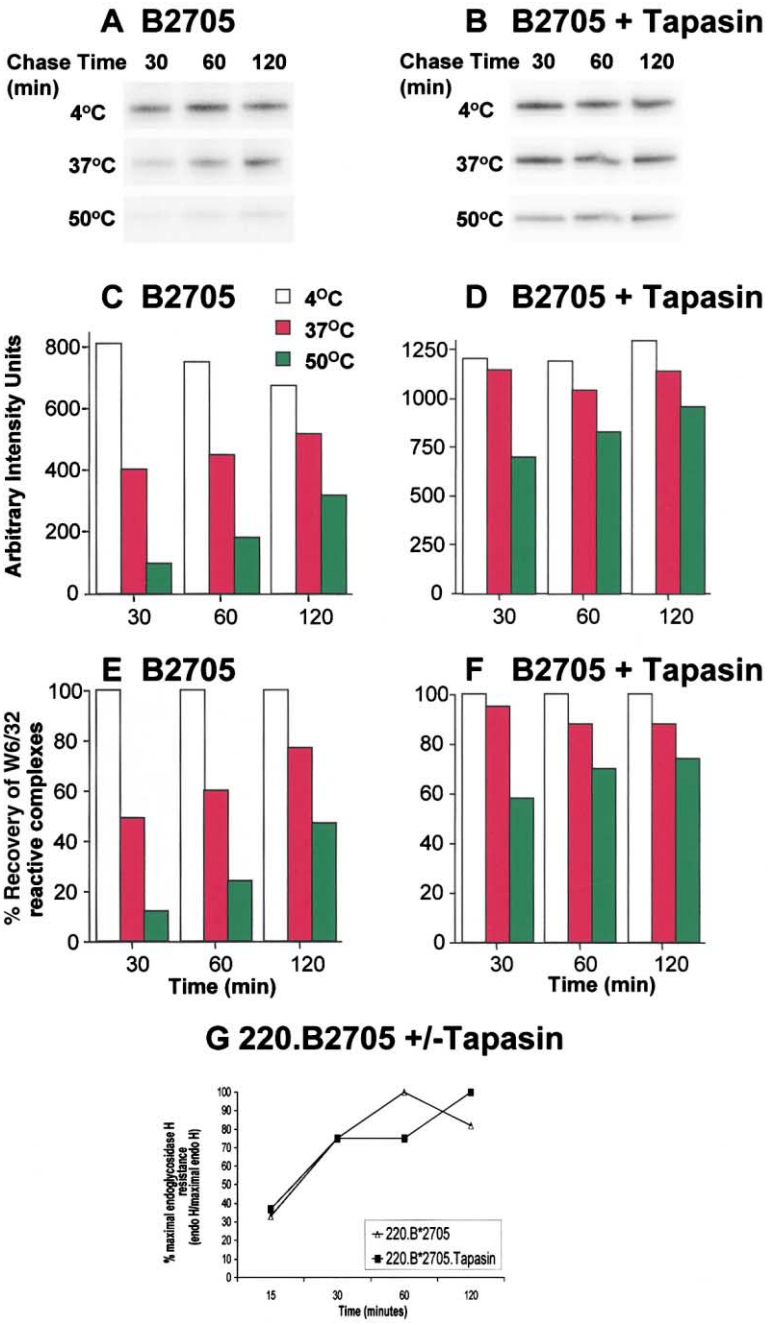

Figure 4. Time-Dependent Optimization of HLA-B*2705 Complexes in Relation to Tapasin

(A–F) .220.B2705 cells in (A), (C), and (E) and .220.B2705.Tapasin cells in (B), (D), and (F) were pulse labeled with [^{35}S]-methionine/cysteine for 5 min and chased in excess cold methionine/cysteine. Equal numbers of cells were harvested at time points indicated. The samples were then processed and analyzed as in Figures 1C–1E. (A)–(F) are representative of three experiments.

(G) 220.B2705 and 220.B2705.Tapasin were labeled for 5 min and chased for 15, 30, 60, and 120 min. At each time point the percentage of endoglycosidase H-resistant material/maximum endoglycosidase H-resistant material is plotted. The results are representative of at least two experiments.

then proceeded to assess the thermostability profiles of B4402 in the presence of either soluble or full-length tapasin (Figure 6). Even though the thermostability of an early cohort of B4402 complexes assembled with soluble tapasin was inferior compared to those assembled with full-length tapasin (Figure 6A), it was evident that soluble tapasin led to an improvement of the peptide cargo of B4402 complexes over time (Figures 6B, 6C, and 6D). However, the extent and rate of optimization mediated by soluble tapasin was reduced compared to the effect of full-length tapasin (Figures 3D and 3F), suggesting that maximal optimization is best achieved with full-length tapasin. This intermediate effect upon the thermostability of B4402 complexes was reflected in faster decay rates of cell surface B4402 in the presence of soluble tapasin compared to B4402 in the presence of full-length tapasin (Figure 6E). Similar results were obtained for B2705 (data not shown). Additionally, we have repeatedly found that B4402 complexes are transported at the same rate in the presence of soluble tapasin compared to full-length tapasin (data not shown). Therefore, early egress from the peptide loading environment of the ER is not a reason for the diminished optimization profiles observed with soluble tapasin. However, it is possible that the diminished optimization seen with soluble tapasin relates to a failure to recruit or promote the function of the other chaperone proteins involved in peptide loading at the TAP interface.

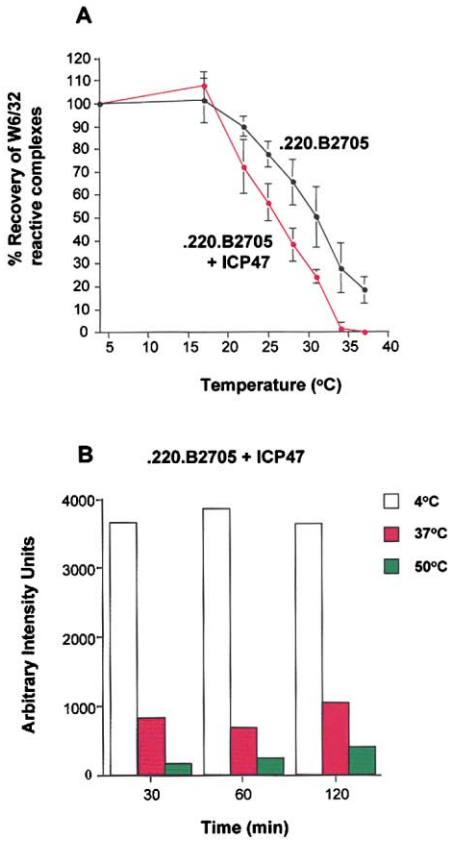

Figure 5. HLA-B*2705 Complexes Are Not Devoid of Peptides in the Absence of Tapasin

(A) .220.B2705 and .220.B2705+ICP47 cells were radiolabeled with [^{35}S]-methionine/cysteine for 30 min and lysed in Triton X-100 buffer. Equal aliquots of lysis supernatant were then processed and analyzed as in Figures 1C–1E. (A) is representative of at least two triplicate experiments.

(B) .220.B2705+ICP47 cells were pulse labeled with [^{35}S]-methionine/cysteine for 5 min and chased in excess cold methionine/cysteine. Equal numbers of cells were harvested at the indicated time points. The cells were then processed and analyzed as in Figures 1C–1E. (B) is representative of two separate experiments.

Thus, we conclude that even though tapasin-mediated class I-TAP interaction is not essential for peptide loading of MHC class I molecules, it is necessary for maximal optimization of the MHC class I peptide complex.

Position 116 Determines the Peptide-Loading Characteristics of B4402

There are 20 amino acid differences that could account for the phenotypic differences in peptide optimization and tapasin dependence between B4402 and B2705. Besides determining peptide binding specificity, these differences may also influence how these class I alleles interact with chaperones that make up the peptide loading complex. Position 116 of MHC class I heavy chain has previously been shown to influence TAP association of certain HLA-B alleles (Neisig et al., 1996). Therefore, we undertook an analysis of the allele HLA-B*4405 (B4405), which is identical to B4402 apart from a polymorphism at position 116. Remarkably, this single amino acid change of tyrosine for aspartic acid at 116 allowed B4405 to be expressed at the cell surface in the absence of tapasin (Figure 7A). The thermostability of B4405 complexes in the presence and absence of tapasin was determined and compared to that of B4402 (Figure 7B). In the absence of tapasin, the stability of an early cohort of B4405 was marginally more thermostable compared to B4402 at all temperatures, with 25% and 10% of its complexes stable at 37°C and 50°C, respectively. Following the introduction of tapasin, there was obvious improvement in the thermostability of recently assembled B4405 complexes. Remarkably, however, the gain in thermostability was much less substantial when compared to B4402 in the presence of tapasin, with only 60% of B4405 complexes attaining 37°C stability (Figure 7B).

In contrast to B4402, B4405 was able to load peptides capable of stabilizing the complex to 50°C in the absence of tapasin (Figure 7C). Similar to B4402, there was significant loss of B4405 complexes over time as indicated by the steep drop in the number of recoverable molecules at 4°C (Figure 7C). Upon the introduction of tapasin, this allele also demonstrated time-dependent and tapasin-dependent optimization of its peptide cargo. However, the extent of optimization for B4405 was inferior compared to that for B4402, with only 65% of B4405 complexes having achieved 50°C thermostability in 2 hr (Figures 7D and 3D). Therefore, it appeared that amino acid residue 116 could determine the tapasin dependence of cell surface expression by permitting a certain degree of peptide optimization in the absence of tapasin. However, like .220.B2705.Tapasin, B4405 is unable to optimize to the same extent as B4402 when tapasin is present. The flexibility gained by using a tapasin-independent pathway may thus be counterbalanced by a less optimal peptide selection compared to a tapasin-dependent pathway.

Discussion

What is Optimization?

Optimization of the peptide repertoire presented by MHC molecules relates to the preferential selection of peptides with slow dissociation rates. In the priming of an immune response against pathogens, foreign peptides will be presented among a plethora of self-peptides on the surface of an antigen-presenting cell (APC). MHC complexes whose cargo of foreign peptides has undergone optimization would have an extended lifespan and thus stand a greater chance of triggering the relevant TCR. Within the MHC class II system, this process of optimization is an obligatory event, with HLA-DM catalyzing exchange reactions in favor of optimal ligands with slow dissociation rates (Weber et al., 1996). Indeed, this mechanism was first recognized using an in vitro assay that utilized SDS stability, rather than thermostability, to differentiate suboptimal and optimal complexes loaded in the presence and absence of HLA-DM (Denzin and Cresswell, 1995). This process of facilitated exchange and kinetic proofreading (Kropshofer et al., 1996) allows for the most stable MHC class II complexes to accumulate on the surface of an APC. For MHC class I, we and others (Cresswell, 2000; Lewis and

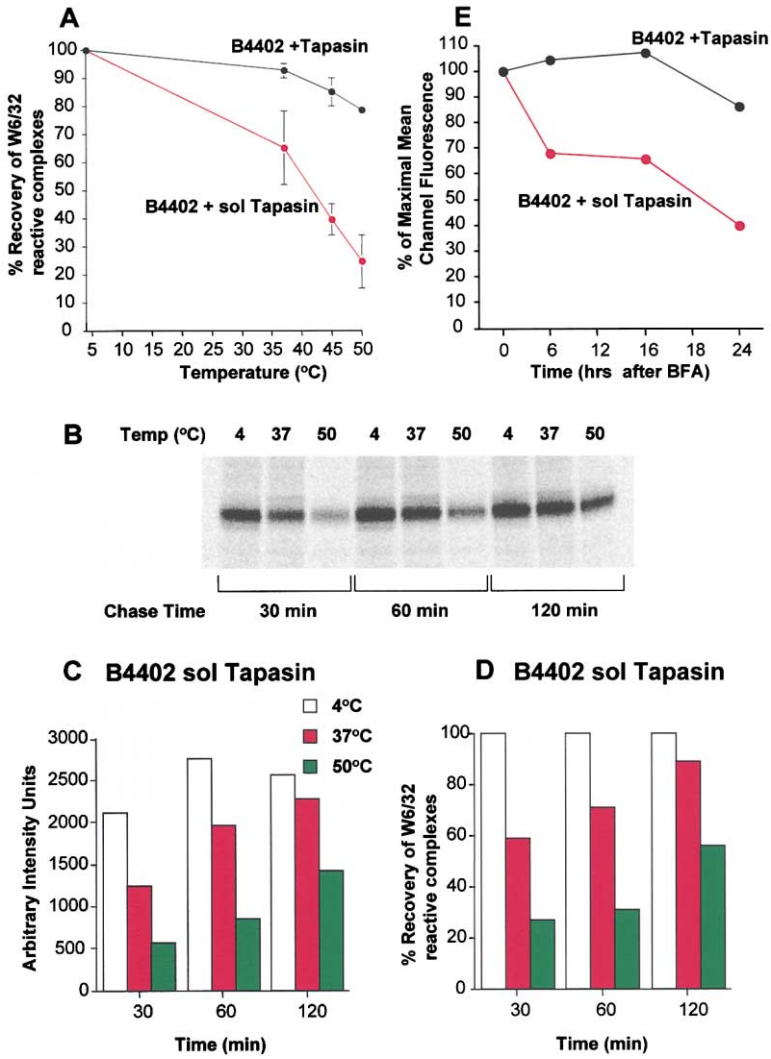

Figure 6. Peptide Optimization of HLA-B*4402 in the Presence of Soluble Tapasin

(A) .220.B4402 and .220.B4402.solTapasin cells were radiolabeled with [^{35}S]-methionine/cysteine for 30 min and lysed in Triton X-100 buffer. Equal aliquots of lysis supernatant were processed and analyzed as in Figures 1C–1E.

(B–D) .220.B4402.solTapasin cells were pulse labeled with [^{35}S]-methionine/cysteine for 5 min and chased in excess cold methionine/cysteine. Equal numbers of cells were harvested at the indicated time points and analyzed as in Figures 1C–1E. (B) Only class I heavy chains are depicted here. (B)–(D) are representative of two separate experiments.

(E) .220.B4402 and .220.B4402.solTapasin cells were treated with brefeldin A (BFA 10 μg/ml) for 30 min to inhibit egress of newly assembled B4402 complexes from the ER. Thereafter, the cells were followed over time in the continuing presence of BFA. At times indicated, equal aliquots of cells were withdrawn and analyzed by FACS for cell surface expression of B4402 with the anti-Bw4 mAb 116.5.28. The level of expression of B4402 was expressed as percentage of mean channel fluorescence at time 0.

Elliott, 1998; Purcell et al., 2001; Sijts and Pamer, 1997) have proposed that an analogous optimization process occurs in class I peptide loading.

Tapasin Catalyzes MHC Class I Peptide Optimization

This study used MHC class I thermostability as a surrogate marker for the changing peptide repertoire of newly synthesized MHC class I alleles over time. In the presence of tapasin, optimization of peptide loading occurred much faster and to a greater extent for both B2705 and B4402. Even though B2705 was capable of tapasin-independent loading, the allele was not entirely tapasin independent, as it could further improve its peptide loading in the presence of tapasin. Although this catalytic effect of tapasin was apparent for both alleles, it occurred with different kinetics and final end points: B2705 optimized quickly between 30–60 min and reached a plateau after 2 hr with 70% of complexes reaching 50°C stability, while B4402 continued to optimize for up to 2 hr, achieving 100% 50°C stability in the process. We believe that this is significant for two reasons. First, it redefines the concept of tapasin independence, and second, it points out that the capacity for peptide optimization without tapasin does not correlate with superior peptide optimization where tapasin is present. Instead, the converse may be true. Tapasin can therefore be considered to have two complimentary effects upon peptide optimization by increasing both the rate and extent of optimization.

The molecular mechanisms mediating the optimization of MHC class I ligands could include peptide exchange and/or peptide trimming. The phenomenon of N-terminal trimming of MHC class I peptide ligands has been well documented within both the ER and early secretory pathway (Brouwenstijn et al., 2001; Serwold et al., 2001; Snyder et al., 1994). It is conceivable that aminopeptidase activity within the ER could trim poor-stabilizing N-extended peptides to an optimal length, thereby stabilizing over time the class I molecule to which they are bound. In addition to ER aminopeptidases, endopeptidases such as furin could also play an important role in optimizing extended MHC class I ligands as the MHC class I peptide complexes traverse the early secretory pathway. The time course of optimization seen for B2705 and B4402 suggests that, while optimization could occur when MHC class I is bound to TAP, some optimization could also occur after dissociation from TAP—as optimization continues for up to 120

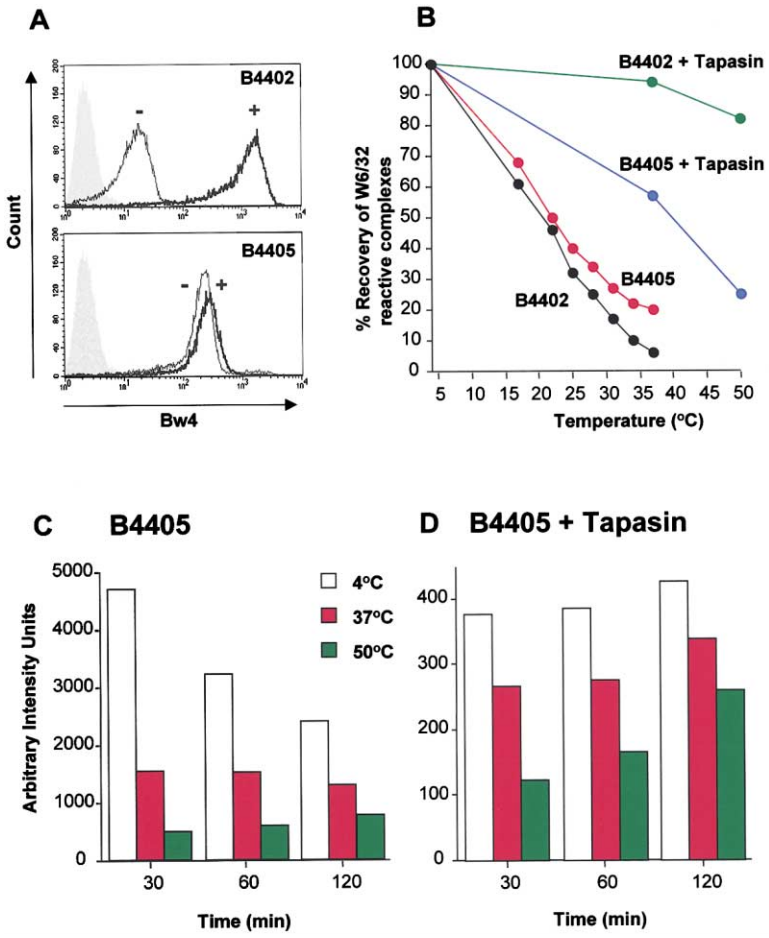

Figure 7. Characteristics of Peptide Optimization of HLA-B*4405

(A) Cell surface expression of B4402 (upper panel) on either .220.B4402 (tapasin −, thin black line) or .220.B4402.Tapasin (tapasin +, thick black line), and of B4405 (lower panel) on either .220.B4405 (tapasin −, thin black line) or .220.B4405.Tapasin (tapasin +, thick black line) was analyzed by FACS with anti-Bw4 mAb 116.5.28.

(B) .220.B4402, .220.B4402.Tapasin, .220.B4405, and .220.B4405.Tapasin cells were radiolabeled with [^{35}S]-methionine/cysteine for 30 min and lysed in Triton X-100 buffer. Equal aliquots of lysis supernatant were processed and analyzed as in Figures 1C–1E.

(C and D) .220.B4405 and .220.B4405.Tapasin cells were pulse labeled with [^{35}S]-methionine/cysteine for 5 min and chased in excess cold methionine/cysteine. Equal numbers of cells were harvested at the time points indicated and analyzed as in Figures 1C–1E. (B)–(D) are representative of two separate experiments.

min for B4402 and 60 min for B2705. Optimization experiments undertaken in the presence of brefeldin A (BFA) showed that the degree of optimization for both alleles was unchanged compared to that undertaken in its absence (data not shown), suggesting that optimization was not dependent upon cell surface expression or endocytic recycling. Additionally, peptide optimization occurred preferentially at the TAP interface since soluble tapasin, which cannot mediate class I bridging to TAP, was suboptimal in this regard. Thus, our experimental data support the concept of optimization occurring within the ER and/or an early secretory pathway with maximal optimization occurring when MHC class I alleles are allowed to load at the TAP interface.

The molecular mechanism of peptide optimization remains unclear. The D to Y substitution at position 116 allowed B4405 to load peptides independently of tapasin and to be expressed at the cell surface at levels comparable to those seen in the presence of tapasin. Position 116 resides in a key position at the base of the F pocket of the peptide binding groove, thereby contributing to peptide selection. It has also been shown to control the extent of the interaction between some MHC class I alleles and the peptide loading complex (Neisig et al., 1996). It is unclear how these properties might be connected. The dependence upon tapasin for loading a repertoire of peptides of sufficient stability to allow egress from the ER may reside in the ability of the F pocket to facilitate the loading of peptides or perhaps to initiate a peptide-induced conformational change in the class I molecule that signals release from the loading complex (Elliott, 1997). Tapasin could regulate this process by stabilizing a peptide-receptive conformation of MHC class I, thereby raising the energy threshold for such a peptide-induced conformational change. This could then lessen the number of class I molecules released from the loading complex bound to peptide ligands with fast off rates. Tapasin could mediate such kinetic proofreading alone or cooperatively with the chaperones calreticulin and ERp57 at the TAP interface. It has been suggested that ERp57 could contribute to optimal peptide selection through an action upon the disulphide bridge between Cys101 and Cys164 within the MHC class I molecule (Cresswell et al., 1999). Similarly, calreticulin may assist in optimization, as cells lacking calreticulin are defective in antigen presentation (Gao et al., 2002). It is therefore possible that the process of MHC class I peptide optimization is dependent upon contributions from all such chaperones at the TAP interface, with tapasin principally involved in recruiting and stabilizing these molecular interactions, thereby facilitating the kinetic proofreading of the assembled MHC class I peptide complexes.

To Load On or Off TAP or Both?

Even though bridging of MHC class I to the TAP complex is important in selecting the most optimal peptides available, alleles like B2705 and B4405 are potentially capa-

ble of loading without tapasin. Hence, our finding that optimization for B2705 was inferior to that of B4402 in the presence of tapasin (Figures 3F and 4F) raises the possibility that simultaneous TAP-independent and TAP-dependent pathways of loading may operate for alleles like B2705 even when tapasin is present, as previously suggested (Neisig et al., 1996). Since our thermostability assay does not distinguish between loading on TAP versus loading off TAP, the optimization profile of B2705 as revealed by our assay may be a composite result of both loading pathways. It is possible that the ability of B2705 to acquire peptides in the absence of tapasin allows a fraction of B2705 to load off TAP and then traffic out of the ER without utilizing the optimization machinery at the TAP interface.

To Optimize or Not?

The capacity of MHC class I ligands to be selected for their slow dissociation rates may be critical in providing a threshold signal for T cell recognition. This process of repertoire optimization may also help to establish a hierarchy of immunodominance among epitopes. In addition to many other factors that determine immunodominance, the biological lifespan of MHC class I complexes has been shown to be important in the selection of a CTL repertoire (van der Burg et al., 1996). It will therefore be intriguing to look at the immunodominance hierarchy of CTLs to pathogens in tapasin knockout mice. Furthermore, tapasin is important for crosspriming, since tapasin-negative DCs show impaired cross-presentation (Garbi et al., 2000). It would also be advantageous for crosspresenting DCs to express MHC class I complexes with long half-lives since the time between capture of antigens in the periphery by DCs and their eventual presentation to T cells in secondary lymphoid organs may take 1–2 days (Banchereau and Steinman, 1998).

The ability of pathogens to subvert the immune response has been studied in detail, and many examples exist regarding interference with components of the MHC class I peptide loading machinery (Fruh et al., 1999). Of particular interest is the adenovirus protein E19 which binds to certain MHC class I molecules, retaining them in the ER and furthermore preventing them from interacting with tapasin and TAP (Bennett et al., 1999). If tapasin is important for the rapid deployment of optimally loaded MHC class I molecules, it might be expected that pathogens would target this mechanism to evade the host CTL response. Similarly, MHC class I alleles, such as B4405, may have diversified so that they may operate independently of this pathway to overcome such subversion. Position 116 is interesting in this regard as it may affect progression to HIV/AIDS (Gao et al., 2001). Indeed HLA-B*3503 is associated with a faster progression to HIV/AIDS compared to HLA-B*3501, and these alleles vary only in position 116. HLA-B*3503 has phenylalanine at position 116 and is known to associate well with TAP, whereas HLA-B*3501 possesses a serine at position 116 and associates poorly with TAP. It will be interesting to see if these individuals possess different CTL reactivities and whether such differences in clinical outcome relate to polymorphisms in antigen-loading pathways. It may be that the TAP/tapasin-independent loading pathway may occasionally be beneficial to some MHC class I alleles in the context of certain infectious diseases.

In summary, we have shown that MHC class I molecules can optimize their peptide cargo over time and that maximum optimization is critically dependent upon tapasin. Furthermore, the degree of optimization is allele dependent and maximal in the presence of full-length tapasin when MHC class I complexes are abridged to TAP. Finally, a single amino acid change at position 116 facilitates a second loading pathway for B4405, allowing it to present peptides at the cell surface in the absence of tapasin. Such MHC polymorphism may ultimately be beneficial to an individual, as the codominant expression of such alleles would maximize the chances of a successful defense against pathogens.

Experimental Procedures

Cell Lines, Antibodies, and Peptides

721.220 is a tapasin-defective human B lymphoblastoid cell line (B-LCL) with homozygous deletions of both HLA-A and HLA-B loci (Greenwood et al., 1994). The transfectants .220.B4402±Tapasin and .220.B2705±Tapasin have been previously described (Peh et al., 1998). The transfectants .220.B4402.solTapasin and .220.B2705.solTapasin were derived from cotransfection of the genes encoding B4402, B2705, and soluble tapasin into .220. The transfectants .220.B4402.ICP47 and .220.B2705.ICP47 were created from transfection of the gene encoding HSV ICP47 (Hill et al., 1995) into .220.B4402 and .220.B2705, respectively. The transfectants .220.B4405±Tapasin were created by cotransfection of the genes encoding B4405±Tapasin into .220. Cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics. W6/32 is a conformation-dependent mouse mAb specific for all HLA class I molecules; mouse mAb 116.5.28 recognizes the HLA-Bw4 epitope (Saxon Europe, UK). Expression of tapasin in relevant transfectants was confirmed by Western blot with a rabbit antisera raised against the N terminus of human tapasin (a gift from B. Gao, University of Oxford). Comparable tapasin expression levels were seen in soluble and full-length transfectants. Peptides were synthesized by F-moc using a Zinnser analytical synthesizer (Advanced Chemtech Inc., Louisville, KY), and purity was confirmed by HPLC.

Gene Constructs

The human full-length tapasin cDNA and soluble tapasin cDNA was cloned into pMCFR.neomycin vector. The ICP47 cDNA was cloned into pMCFR.puromycin vector. Genomic B4402 and B2705 were cloned into puc13 vector as previously described (Peh et al., 1998). B4402 cDNA was mutated to B4405 by site-directed mutagenesis using the 5′ forward primer GGTATGACCAGTACGCCTACGAC and the 3′ reverse primer CCATACAGGTCATGCGGATGCTG (Quikchange Site-Directed Mutagenesis, Stratagene). These cDNA constructs were cloned into the RSV.5neo vector. 721.220 cells were transfected by electroporation at 220V and 960 μF with the relevant constructs and selected with G418 0.5 mg/ml and/or puromycin 0.4 μg/ml. Where possible, positive transfectants were sorted for peak channel expression of HLA molecules using magnetic beads that had been coated with an appropriate mAb.

Thermostability Assays

For thermostability assays, the relevant cell transfectants were starved in methionine- and cysteine-free medium for 40 min and pulsed with [^{35}S]-methionine/cysteine (Promix, Amersham, UK) at 100 μCi/ml/10^7 cells for 30 min. Cells were collected, washed in cold PBS, and lysed in Triton X-100 buffer (1% Triton X-100, 10 mM Tris [pH 7.4], and 150 mM NaCl with PMSF) for 30 min at 4°C. To test for stabilization of MHC class I complexes by variant peptides, this incubation step was carried out in the presence of 50 μM of the relevant peptide. After cell nuclei had been centrifuged, cell lysates were precleared with protein A-sepharose beads (Sigma,

St. Louis, MO) for 30 min. Following this, equal aliquots of cell extracts (derived from 2×10^6 cells) were loaded into thin-walled eppendorfs and kept at either 4°C or placed across a temperature gradient in a thermocycler (Mastercycler Gradient, Eppendorf). The samples were heated at required temperatures for 12 min and then returned immediately to 4°C. Thereafter, mAb W6/32 and protein A-sepharose beads were added for 1 hr at 4°C with gentle rotation. The beads were washed once in 450 mM NaCl solution containing 0.1% Triton X-100 and 10 mM Tris buffer (pH 7.4) and once in 10mM Tris buffer (pH 7.4). The beads were heated at 80°C in 20 μl of ×2 LDS sample buffer (pH 8.5) for 5 min, and proteins were subsequently separated by 10% nonreduced SDS-PAGE gel. The gel was then fixed, soaked in Amplify (Amersham, UK), and dried. The images were analyzed with a Fujifilm FLA-2000 phosphorimager. The arbitrary intensity units (AIU) of each heavy chain signal were quantified, and the relative stability of class I complexes after heating is expressed as the AIU after heating in relation to the relevant AIU at 4°C. For pulse-chase thermostability assays, cells were labeled for 5 min with [^{35}S]-methionine/cysteine at 100 μCi/ml/10^7 cells and then chased in excess methionine (2 mM) and cysteine (2 mM). At indicated time points, cells were harvested and lysed in Triton X-100 buffer. Equal aliquots of cell extracts derived from 2×10^6 cells were kept at either 4°C or heated at indicated temperatures for 12 min. Thereafter, samples were treated as above. Where cell extracts had been heated to 50°C, the samples were spun at 21,000 g for 10 min to remove any heat-induced precipitates prior to immunoprecipitation.

Pulse-Chase Radiolabeling

Cells were starved in methionine- and cysteine-free medium for 40 min and pulsed with [^{35}S]-methionine/cysteine (Promix, Amersham, UK) at 100 μCi/ml/10^7 cells for 5 min. The cells were then chased for 15, 30, 60, and 120 min in RPMI 1640 media supplemented with 10% FCS and 2 mM methionine/cysteine. At each time point, cell aliquots were removed, washed in ice-cold PBS, and lysed in 1% Triton X-100 buffer (pH 7.4). Postnuclear supernatants were precleared for 1 hr at 4°C prior to immunoprecipitation with W6/32 conjugated sepharose beads for 1 hr at 4°C. Washed precipitates were resuspended in 20 uL of 50 mM sodium citrate buffer (pH 5.5) containing 0.2% SDS, heated to 80°C for 5 min, and digested with 5 mU endoglycosidase H (Roche) overnight at 37°C. Samples were analyzed on a 10% SDS-PAGE gel. The gel was then fixed, soaked in Amplify (Amersham, UK), and dried. The images were analyzed with a Fujifilm FLA-2000 phosphorimager. The data was then plotted as the percentage of maximal endoglycosidase H-resistant material seen at each time point.

Cell Surface Radio-Iodination

Cells (1×10^6) were washed once in Dulbecco's PBS (Sigma, St. Louis, MO) and radiolabeled with 0.5 mCi 125-iodine in 1 ml of Dulbecco's PBS within an Iodo-Gen precoated tube (Pierce) for 15 min at room temperature with gentle agitation. Cells were then washed twice in Dulbecco's PBS containing 5 mM KI, lysed in Triton X-100/TBS buffer, and subjected to immunoprecipitation as described above.

Flow Cytometry

Cells were stained with mAb 116.5.28 for HLA-B4402 followed by goat anti-mouse antiserum conjugated to FITC (Sigma, St. Louis, MO). 2×10^4 cells were analyzed for each histogram using a FACS analyser (Becton Dickinson, CA). For cell surface decay experiments, 5×10^6 cells were treated with 10 μg/ml BFA and cultured at 37°C. Equal aliquots of cells were withdrawn at 0, 8, 16, and 24 hr, stained with mAb, and fixed with 1% paraformaldehyde in PBS. Cell surface decay of class I complexes was expressed as mean channel fluorescence (MCF) at these time points in relation to the MCF at time 0.

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